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This was a study to determine whether cytomegalovirus (CMV) might be a cofactor with the human immunodeficiency virus type 1 (HIV-1) to accelerate immunosuppression. Over seven hundred healthy HIV-1-seropositive U.S. Air Force personnel consented to be a part of this study, of whom 6.3% were initially CMV-seronegative. The CMV-seroconversion rate among seronegatives was 19%/yr, indicating primary infection through close personal contacts. Three groups of subjects were studied to compare their rates of CD4+T-cell decline: CMV-seronegatives, CMV-seropositives, and CMV-seroconverters. CD4+T-cell depletion in CMV-seropositive subjects whose cell numbers were decreasing has been significantly faster than in CMV-seronegatives ($P = 0.003$). Using a similar means of analysis, herpes simplex virus type 2 (HSV-2) infection was not found to be associated with decline in CD4+T-cell depletion in HIV-1-infected people. The variations in the rates of CD4+T-cell decline among CMV-seropositive subjects were significantly greater than among CMV-seronegative subjects ($P \leq 0.0001$). CMV neutralizing antibody is produced efficiently in WR-5 and 6 subjects, who also produce CMV-specific IgM. CMV isolates are almost identical antigenically.

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KOS For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

n/a In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Kendall O. Smith Date 12/06/91

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## INTRODUCTION

Numerous studies have shown that nearly all AIDS patients are CMV-infected (1-3), that CMV latency often shifts to active infection (4-5), with consequent severe disease in HIV-immunosuppressed people (6-9), and that active CMV-infection itself is immunosuppressive (10-13). These phenomena make it difficult to determine whether there is a "triggering" cofactor role in dual CMV-HIV infection, i.e., whether the synergistic phenomena well documented in doubly infected cells *in vitro* (12,14,15) have any relevance to the clinical course of doubly infected people.

Individual brain cells in AIDS patients are coinfected with CMV and HIV (7,8), which suggests that CMV could modify the pathogenesis of HIV infection, at least in the brain. CMV genomes can be found in both polymorphonuclear and mononuclear human leukocytes, including OKT4 cells. Therefore, dually infected OKT-4 cells are probably present in many HIV-infected subjects and the two viruses might interact *in vivo* in a manner similar to that observed *in vitro*.

The published evidence for the possibility that CMV infection serves as an early, triggering cofactor in AIDS is, not surprisingly, small. At present there seems to be little direct evidence that the *in vitro* interactions between CMV and HIV have any relevance in patients. Perhaps the best published evidence that CMV-infection is associated with a more rapid progression to HIV disease is the report of Webster et al. (35), who studied the effect of CMV and herpes simplex virus (HSV) infection in 108 HIV-infected hemophiliacs. They found that the risk for development of AIDS in CMV-seropositive subjects was 2.5-fold greater than in CMV-seronegative subjects. Seropositivity to HSV was not associated with AIDS progression, making the interpretation of their analysis and their conclusions seem more valid.

Others have encountered difficulty in determining whether CMV infection may accelerate immunosuppression in HIV-infected subjects because most HIV-infected subjects are also CMV-infected, therefore very few CMV-negative, HIV-positive subjects exist for study. Nearly all of the HIV-seropositive Air Force personnel in our

study were physically and immunologically normal, suggesting early stage HIV infections. Many were serologically negative on first screening and seroconverted during this study. This presented interesting opportunities for us to study the possible triggering cofactor (early) role of CMV in subjects who were likely to have been recently infected with HIV, not already clinically immunosuppressed. Of great importance to this study was the fact than about 6% of the HIV-seropositive subjects were CMV-seronegative, which gave us the opportunity to follow these subjects and compare their clinical course with 94% who were dually infected on entry into the study. Some of the CMV-negative subjects seroconverted during the course of the study, presenting an interesting third group to study. The fact that a relatively large number of subjects was involved (over 400 who visited at least twice) possibly makes our study more valid than smaller studies from the statistical point of view.

## EXPERIMENTAL METHODS AND RESULTS

### Cell culture and viral strains

MRC5 cells were obtained from Whittaker Bioproducts (Walkersville, MD) and were grown in Basal Medium Eagle (Flow Labs, Dublin, VA) with 10% calf bovine serum. The Towne strain of CMV (American Type Culture Collection, Rockville, MD) was used in all the CMV Western blot (WB) studies, HSV-1 (KOS strain) and G strain of HSV-2 (American Type Culture Collection) were used for all HSV Western blot studies.

### Antigen production

CMV was grown in a 50-60% monolayer of MRC5 cells and harvested when 100% cytopathic effect was seen. To harvest the antigen, the cells were gently scraped from the T150 (150 $cm^2$) plastic flask and centrifuged at approximately 400 x *g* for 10 min. The cells were washed with 50 ml of phosphate buffered saline (PBS), centrifuged as above and the pellet was resuspended in 1 ml of PBS (to give a 50x concentration). This was performed on each T150 flask. The antigen aliquots were frozen at -70°C, thawed when needed and sonic oscillated for 3 min before being separated by gel electrophoresis.

HSV was inoculated onto an 80% monolayer of MRC5 cells and harvested at approximately 48 hr (100% cytopathic effect was seen). The procedure for harvesting and storing HSV-1 and HSV-2 was the same as described for CMV antigen.

### WB procedure

CMV or HSV antigens were separated by gel electrophoresis by the method described by Laemmli [18]. The separated proteins were then electrically transferred to nitrocellulose as described by Burnette [19]. The nitrocellulose blots were cut into

2.5 mm width strips. These strips were "blocked" (excess non-specific proteins added) for 10 min with 3.75% milk (Carnation dry non-fat milk) in isotonic PBS (which is called blotto, pH = 7.4) [20]. Dilutions (1:100 for CMV antigen-treated strips and 1:50 for normal cell antigen-treated strips) of the subjects' plasma samples were made in blotto and the strips were left in the dilutions for 1 hour with shaking. The strips were rinsed X 3, transferred to clean trays and left in deionized (di)$H_2O$ for 15 min. A 1:100 solution of labeled anti-human IgG, γ-chain specific, affinity purified antibodies conjugated with horseradish peroxidase (obtained from Calbiochem, San Diego, CA), diluted in blotto was placed over the strips and incubated for 1 h. Another X3 di$H_2O$ rinse followed and the strips were transferred to clean trays and left with di$H_2O$ on them for 15 min. Finally the water was removed and the strips were developed with the substrate 4-chloro-1-naphtol (Sigma, St. Louis, MO).

Interpretation of WB's

In order to interpret the results of WB's used to test samples for CMV antibodies, we developed guidelines based upon published reports [21,22] and after having examined several hundred strips. It was noted that there were 3 bands which were common in almost all of the obviously positive strips. We considered the presence of any of these "major" bands (140, 48 and 34 Kd) to indicate a CMV-seropositive sample. However, in most cases 2 or all 3 of the major bands were easily recognized in WB's of CMV-seropositive samples. If any other bands were present while major bands were absent, and these "minor" bands matched the bands on a cellular antigen strip (on which electrophoresis was done simultaneously), the sample was considered CMV-seronegative. If any minor bands were present on the CMV antigen strip and there were no corresponding bands on the cellular antigen strip, the sample was labeled "indeterminant." Only 8 of over 1,200 samples had to be classified indeterminant. All the other subjects were classified by WB into 3

groups: group 1 - CMV-seronegatives; group 2 - CMV-seropositives; and group 3 - CMV-seroconverters (seroconverted during the course of the study).

Regarding HSV-1 and HSV-2 antibody status, we noted the characteristic banding patterns with known control sera and devised guidelines similar to those above for making determinations. Samples tested for HSV-1 were considered positive if either or both 140 and 45 Kd bands were present on nitrocellulose strips with HSV-1 antigens. Samples tested for HSV-2 were considered positive if 2 or more of these bands were present on nitrocellulose strips with HSV-2 antigens: 55, 50 or 45 Kd. This approach to identifying banding patterns for differentiating HSV-1 and 2 antibody reactions was similar to the procedures described by Bernstein et al. [23], although our molecular weight determinations of the bands were slightly different from those reported by Bernstein et al.

Plasma samples

Over 700 different subjects' blood samples containing EDTA anticoagulant were taken at Wilford Hall Medical Center (WHMC, Lackland Air Force Base, San Antonio, Texas) and the plasma was separated at the University of Texas Health Science Center (UTHSC). Each plasma was aliquotted and frozen at -70°C until used. In view of our use of calcium - containing diluent in all assays (blotto), the plasma was converted to serum upon dilution and is therefore referred to hereafter as serum. No troublesome clotting was encountered at the plasma dilutions used.

Procedure for determining changes in CD4 + T-cell concentration (number/$mm^3$)

The CD4 + T-cell number was determined on blood collected at about 7:30 AM, which may have helped avoid diurnal variations [24]. Flow cytometry was done at WHMC, as previously described (Clerici et al. [25]). CMV WB antibody evaluations were done at the UTHSC on serum samples which had been collected from the subject

at the same time. A blood sample was received from each subject for CD4+T-cell count and CMV WB on an average of every 13 months. The only subjects selected for this study were those classified as Walter Reed (WR) 1 or 2 at the time of entry into the program. The WR staging evaluation-classification of each subject was done at WHMC and followed the protocol described by Redfield et al. [26]. Each of the subjects included in the study reported here returned to WHMC for reassessment-medical evaluation at least once. CD4+T-cell numbers obtained on a subject within 6 months of each other were not considered; multiple CD4+T-cell number determinations within one month were averaged.

We asked the question: "among subjects whose CD4+T-cell numbers were declining, were there significant differences in the rates of CD4+T-cell decline between the 3 CMV-serologic groups?".

All of the subjects in the cohort classified as WR 1 or 2 whose CD4+T-cell numbers were declining at the last visit (compared with the highest number found at any previous visit) were included for analysis. These subjects were then placed in CMV serologic groups 1, 2 or 3, as indicated above, on the basis of WB analysis. The CD4+T-cell number at the last visit was subtracted from the highest number obtained at any prior visit and the rate of decline was calculated (giving the cell number decline/time interval between these visits). This rate of decline was then normalized to decline/12 months. Then the mean ($\bar{x}$) rate of decline and standard deviation(s) for each group were calculated and Student's t-test was used to determine whether the rates of decline were significantly different between the groups. T-tests were computed taking unequal variances into account when appropriate (due to differences between the groups in standard deviations; see Results section) and a two-tail chart was used to determine the level of significance. Stat-sak, "The Statisticians Swiss Army Knife", was the computer software used to determine all t-test values. Since only CD4+T-cell declining subjects were included

for decline rate calculations, a one-tail chart might appropriate. However, we used a two-tail chart in order to interpret the results conservatively.

Chi-square and Fisher's exact caluclations were also made using Stat-sak. Results were two-tailed.

Other serologic test

FIAX tests were performed at WHMC for detecting CMV, HSV-1, and HSV-2 antibodies. FIAX is a commercially produced fluorescence system (Whittaker M.A. Bioproducts, Walkersville, MD).

Method for calculating the rate (incidence) of CMV-seroconversion

The number of CMV-seroconverters was divided by the total number of CMV-seronegatives per unit time. Six subjects who seroconverted, and the time interval between blood sample testing was 18 months or more, were not included in these calculations. Seroconversion rate was calculated (normalized) to percent seroconversion per 12 months.

Description of subject population

The U.S. Air Force mandatory HIV-1-screening between 1985 and 1990 yielded approximately 1000 HIV-1-seropositive persons. Most of this cohort were asymptomatic. Fifty-five percent (55%) of the subjects were Caucasian, 39% Black and 7% Hispanic, Asian or of other racial groups; 4.5% have been female. From this cohort, 773 had volunteered to be a part of this study by July, 1990 and this is the larger pool from which subjects were taken.

The subjects were an average of 29 years old ($\sigma = 5.7$) upon entry into the study and the average length of time each subject had spent in the study was 32 months; the shortest amount of time was 9 months and the longest was 52 months. None of

the subjects admitted into this study were taking azidothymidine (AZT) at any time before or during the study.

There was a total of 509 subjects who visited WHMC at least twice for medical evaluation and volunteered blood samples at the time of their visits. At the time of this writing, 26 of these were CMV-seronegative, 465 were seropositive and 11 were seroconverters (see Table 1). The remaining 7 were labelled indeterminant (see Results section). Of the 439 subjects who had visited Wilford Hall at least twice and were classified WR1 or 2 at time of entry into the program, 20 were CMV-seronegative, 409 were seropositive and 10 were seroconverters. There were 317 subjects who had visited WHMC at least twice, were WR1 or 2 at time of entry and whose CD4+T-cell numbers were declining. Twelve of these 317 were CMV-seronegative, 298 were seropositive and 7 were seroconverters.

The major purpose of this study was to compare the following three groups of subjects, all of whom were HIV seropositive:

Group 1 - CMV-seronegatives;

Group 2 - CMV-seropositives; and

Group 3 - CMV-seroconverters (during the study).

Therefore, plasma from each subject in the study was collected at each time they visited Wilford Hall Medical Center, the plasma was tested for antibodies to CMV, and the subject was placed appropriately in one of the three study groups. The CD4+ T-cell number and the WR classification of each subject was also determined each visit to the hospital. At about quarterly intervals, the mean CD4+ T-cell number change was determined for each group, as well as the mean change in WR classification. Statistical analysis was done to determine whether CMV-serologic status, reflecting presence or absence of CMV-HIV coinfection, was related to rate of CD4+ T-cell decline and disease progression. Since this required slow, systematic collection and storage of plasma, cell counting serologic re-evaluation and clinical

evaluation over a period of several years, we attempted to address some other questions regarding CMV-HIV coinfection which might clarify the possible role of CMV as a triggering cofactor in AIDS progression. The results of addressing the major purpose of the study are given completely in the mid-term report, dated 8/30/91. CD-4 T-cell numbers decreased at a faster rate in CMV seropositive subjects than in CMV seronegative subjects.

Some of the following secondary questions were asked, while the more time consuming major-purpose was being considered:

1. Were serum antibodies from subjects who had progressed to higher WR stages less able to neutralize CMV *in vitro*? β-2-microglobulin is increased in HIV infected subjects who are progressing rapidly (Moss et al., 1988), binds to CMV and might inhibit its neutralization (Grundy et al., 1988). Also, CMV infection can increase β-2-microglobulin production (Hutt-Fletcher et al., 1983). Another possibility is that *in vitro* reactive CMV-antibodies (e.g., that detected by WB) may not necessarily have good antiviral neutralizing capabilities (i.e., biological activity) because of the highly specific nature of antibody neutralization of viruses. Thus, the biological functions of CMV-specific antibodies might be compromised in subjects rapidly progressing to WR-6 even though the quantities of non-neutralizing antibodies (detectable by WB) remained relatively unaffected. The results of these studies showed little significant difference between subjects with early infection (WR-1 and 2) and clinically advanced subjects (WR-6) in their abilities to produce biologically functional (neutralizing) CMV-antibodies. The ratio of WB titers to virus neutralizing titers was 171 (WB titration being more sensitive).
2. Is CMV-specific IgM more prevalent or in higher concentrations in clinically advanced subjects compared with healthy subjects? The answer

to this question was "yes". Higher concentrations of CMV-specific IgM were found in WR-6 patients than were found in WR-1 subjects ($p = 0.005$). This was not surprising, having been observed by others; recrudescence of latent CMV infections is well known in AIDS patients and CMV induced diseases are well known in profoundly immunosuppressed subjects. Obviously, such immunosuppressed subjects can still make enough CMV-specific anti-IgM to be measurable by WB. The results of studies on secondary questions 1 and 2, along with studies on cell-specific antibodies found in HIV-infected subjects sera, are shown in Appendix 2. These results, not remarkable, confirm that advanced subjects frequently have subclinical recrudescence of latent CMV infections; somewhat surprising is that the humoral immune system can still function to produce sufficient virus-specific IgM to be detected by WB.

3. The question of whether epidemiologically unrelated CMV isolates are very different from each other in gene products (banding patterns by WB) was addressed. Over 20 different CMV isolates were grown in cell cultures sufficiently to allow coelectrophoresis with other strains and with Ad-169 and Towne strains for comparison. The first results comparing different strains treated with the same serum antibody showed remarkably <u>few qualitative</u> differences in strains, although strains varied considerably quantitatively in their <u>concentrations</u> of certain banded proteins, i.e., intensity of certain bands). However, if genetically different strains (most new isolates are) sometimes produce unique protein bands, the serum antibody from the subject from whom a particular isolate came would have to be used to detect that unique protein. Therefore, we studied many isolate-antibody combinations from the same subjects by WB for evidence of unique bands. None could be identified. It was clear that

bands "missing" with one isolate were frequently present in many other isolates and that passage of the virus in cell cultures could give widely differing relative concentrations of banded proteins in different cell culture passages of the same viruses. It is concluded that, although epidemiologically different CMV isolates clearly show distinct genome differences, these are not reflected in significant differences in the gene products observed in western blots. Some reports suggesting strain differences observable by WB probably reflect quantitative differences in proteins produced under different cell culture passage conditions with the same virus. One published report claiming strain differences in protein banding by WB involved the illogical strategy of using sera from subjects other than those from whom the strains were isolated in order to show unique bands by WB. A practical correlary of the results we obtained suggests that either the Ad-169 or the Towne strains (laboratory prototype strains) could be used effectively to do WB studies for CMV-specific antibodies on all human sera. We, therefore, followed this practice throughout our studies.

4. The question arose, which serological test would be most reliable to detect CMV-specific antibodies in subjects' sera? We compared the Wittaker ELISA, the FIAX, the slide ELISA, the bead ELISA (Organon) and the WB as a "gold standard" to determine relative sensitivities and specificities of these tests. The sensitivities of all tests were adequate but the specificities were a real problem with all but WB. The high prevalence of antibodies to normal, uninfected cells caused non-specific "noise" in all tests except the WB, which allowed differentiation of antibodies to cellular proteins from CMV-specific proteins by banding patterns. The result of our using the WB for routine serologic testing in all of our studies was that

we were able to identify some CMV-seronegative subjects who would have been classified seropositive by some of the other methods. These results with CMV serology are similar to those seen with HIV serology, where sometimes up to 80% of ELISA-seropositive sera are not confirmed by WB (depending upon the purity of the HIV antigens, this figure can be smaller). False positive results may explain why some have reported 100% CMV seropositives among AIDS patients.

During the process of studying the sensitivity and specificity characteristics of these various serologic tests, we were able to obtained exquisitely purified HIV antigens from Organon-Technica (at no cost) to test some sera known to have high concentrations of normal cell-specific antibodies by WB. By using these HIV antigens in a slide ELISA (see Appendix 3), we were able to show a high sensitivity and specificity with hundreds of randomly selected HIV-positive and HIV-negative sera. This test has attracted attention in several developing countries and is being manufactured and field-tested in Tanzania, Central Africa, on a large scale. The Principal Investigator devised an inexpensive ($120), portable, reliable vertical-beam colorimeter, for reading this test, that can be assembled from easily available parts by the user. The above studies demonstrated the importance of using highly purified antigens for doing serologic studies by ELISA. Alternatively, the WB allows identification of characteristic banding patterns for differentiating virus-specific from non-specific reactions <u>if</u> normal cell control strips are run in parallel (we did in all of our studies).

5. The <u>incidence</u> of primary CMV infection in seronegative subjects was determined as a secondary effort in this study. The CMV seroconversion rate (incidence of primary infection in naive subjects) was about 23% per

year. This, of course, rapidly reduced our pool of CMV-seronegative, HIV positive subjects over the course of these studies. The high incidence of CMV-sero-conversion in HIV-positive, CMV-seronegative subjects demonstrates that Air Force personnel who are HIV infected continue to practice intimate physical contact with others, probably exchanging body fluids which transmit CMV (and sometimes HIV) strains other than those of the recipients'.

6. Would centrifugation upon a flat surface of MRC-5 cells improve the recoverability of CMV from human specimens such as saliva and urine? The answer was "yes". In comparative studies with known concentrations of Towne strain of CMV, the sensitivity of virus detection was increased by about 10-fold by this procedure (described in earlier reports). However, there seemed to be an increase in the number of bacterially contaminated cultures and an increase in the amount of debris on the cell sheet, which could sometimes not be removed by gentle rinsing. On balance, centrifugation was considered an improvement and resulted in isolation from a higher percentage of cultures which were maintainable to the end (18-21 days). This higher frequency of isolation by centrifugation-inoculation probably more accurately reflected the true pattern of very frequent CMV shedding in most HIV-seropositive subjects. This high frequency of shedding throughout the WR stages led us to the conclusion that this was not a very useful marker for HIV progression, although the frequency of viral isolation from salivas of advanced subjects was somewhat greater than in WR-1 subjects (see previous reports).

7. How long are electrophoresed, cellulose paper blotted, dried CMV and cellular antigens stable on storage at room temperature? The answer was "excellent stability, longer than one year". This enabled us to produce WB

strips whose quality could be highly controlled, which made it possible to get uniform, comparable WB results over a long period of time. A part of this quality control was the routine use of strong CMV-positive and negative reference sera in every WB assay run performed. By storage of small volumes of diluted serum controls, the same controls could be used over many months for all WB assays. A permanent photographic record was made of the finished blots so that fading would not compromise later analysis of data.

8. Does the CMV-WB IgG or IgM banding pattern in WR-1 subjects differ from the pattern seen in WR-6 subjects? Hundreds of hours were spent comparing WB banding patterns in several different ways to see if they reflected changes in clinical status. This was done qualitatively and quantitatively, scoring bands on a scale of neg. to 4+. No statistically significant differences could be found in CMV-IgG, so we concluded that CMV-IgG banding patterns were not a reliable marker for AIDS disease progression. As indicated elsewhere, however, the frequency of strong CMV-specific IgM banding was higher in later stage (WR-5,6) subjects than in WR-1 stage subjects.

9. Could the WB technique be used to detect CMV antigens directly in the same urine of subjects from whom CMV was isolated by cell culture. The answer was "no". Several (10) undiluted urines giving positive culture results and several giving negative results were treated as if they were cell culture grown CMV specimens, electrophoresed and western blotted with CMV antibodies to see if they would or would not demonstrate characteristic banding patterns. No CMV bands could be detected in any urine specimens.

**Table 1. Description of the subject population**

| Subject status | Numbers of subjects in various CMV study groups | | | |
|---|---|---|---|---|
| | Group 1* | Group 2* | Group 3 | Total |
| Classified WR1 → 6† | 26 | 465 | 11 | 502 |
| Classified WR1 or 2† | 20 | 409 | 10 | 439 |

* Mean CD4+T-cell numbers on entry were: group 1 - 624; group 2 - 717; there was no significant difference between these means ($\underline{P} = .117$, using Student's $\underline{t}$-test).

† Visited WHMC at least 2X, medically evaluated, volunteered blood samples at time of visit.

## REFERENCES


1. Halbert SP, Kiefer DJ, Friedman-Kien AE, Poiesz B. Antibody levels for cytomegalovirus, herpes simplex virus, and rubella in patients with acquired immune deficiency syndrome. J Clin Microbiol 1986; 23:318-21
2. Drew WL, Mintz L, Miner RC, Sands M, Ketterer B. Prevalence of cytomegalovirus infection in homosexual men. J Infect Dis 1981; 143:188-92
3. Cheeseman SH, Sullivan JL, Brettlar DB, Levine PH. Analysis of cytomegalovirus and Epstein-Barr virus antibody responses in treated hemophiliacs. JAMA 1984; 252:83-85
4. Buimovici-Klein E, Lange M, Ong KR, Grieco MH, Cooper LZ. Virus isolation and immune studies in a cohort of homosexual men. J Med Virol 1988; 25:371-385
5. Handsfield HH, Chandler SH, Caine VA, Meyers JD, Corey L, Medeiros E, McDougall JK. Cytomegalovirus infection in sex partners: evidence for sexual transmission. J Infect Dis 1985; 151:344-48
6. Drew WL. Cytomegalovirus infection in patients with AIDS. J Infect Dis 1988; 158:449-56
7. Wiley CA, Nelson JA. Role of human immunodeficiency virus and cytomegalovirus in AIDS encephalitis. Am J Path 1988; 133:73-81
8. Nelson JA, Reynolds-Kohler C, Oldstone MBA, Wiley CA. HIV and HCMV coinfect brain cells in patients with AIDS. Virol 1988; 165:286-90
9. Armstrong D, Gold JWM, Dryjanski J, Whimbey E, Polsky B, Hawkins C, Brown AE, Bernard E, Kiehn TE. Treatment of infections in patients with the acquired immunodeficiency syndrome. Ann Intern Med 1985; 103:738-43

10. O'Toole CM, Gray JJ, Maher P, Wreghitt TG. Persistent excretion of cytomegalovirus in heart transplant patients correlates with inversion of the ratio of T-helper/T-suppressor-cytotoxic cells. J Infect Dis 1986; 153:1160-62

11. Rinaldo CR Jr, Carney WP, Richter BS, Black PH, Hirsch MS. Mechanisms of immunosuppression in cytomegaloviral mononucleosis. J Infect Dis 1980; 141:488-95

12. Hirsch MS, Felsenstein D. Cytomegalovirus-induced immunosuppression. Ann NY Acad Sci 1984; 437:8-15

13. Sirianni MC, Testi R, Bonomo G, Aiuti F. Lack of specific cell mediated immunity to cytomegalovirus in people at risk for AIDS. AIDS Research 1984; 1:99-105

14. Skolnick PR, Kosloff BR, Hirsch MS. Bidirectional interactions between human immunodeficiency virus type 1 and cytomegalovirus. J Infect Dis 1988; 157:508-14

15. Davis MG, Kenney SC, Kamine J, Pagano JS, Huang E-S. Immediate-early gene region of human cytomegalovirus trans-activates the promoter of human immunodeficiency virus. Proc Natl Acad Sci USA 1987; 84:8642-46

16. DeWolf F, Roos M, Lange JMA, Houweling JTM, Coutinho RA, Van DerNoordaa J, Schellekens PT, Goudsmit J. Decline in CD4+ cell numbers reflects increase in HIV-1 replication. AIDS Res Hum Retroviruses 1988; 4:433

17. Giorgi JV, Detels R. T-cell subset alterations in HIV-infected homosexual men: NIAID multicenter AIDS cohort study. Clin Immunol Immunopathol 1989; 52:10-18

18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. Nature 1970; 227:680-85

19. Burnette WN. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 1981; 112:195

20. Johnson DA, Gautsch JW, Sportsman JR, Elder JH. Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Tech 1984; 1:3-8

21. Landini MP, Guan MX, Jahn G, Lindenmaier W, Mach M, Ripalti A, Necker A, Lazzarotto T, Plachter B. Large-scale screening of human sera with cytomegalovirus recombinant antigens. J Clin Microbiol 1990; 28:1375-79

22. Landini MP, Re MC, Mirolo G, Baldassarri B, La Placa M. Human immune response to cytomegalovirus structural polypeptides studied by immunoblotting. J Med Virol 1985; 17:303-11

23. Bernstein DI, Bryson YJ, Lovett MA. Antibody response to type-common and type-unique epitopes of herpes simplex virus polypeptides. J Med Virol 1985; 15:251-63

24. Malone JL, Simms TE, Gray GC, Wagner KF, Burge JR, Burke DS. Sources of variability in repeated T-helper lymphocyte counts from human immunodeficiency virus type 1-infected patients: total lymphocyte count fluctuations and diurnal cycle are important. J Acquir Immune Defic Syndr 1990; 3:144-51

25. Clerici M, Stocks NI, Zajak RA, Boswell RN, Lucey DR, Via CS, Shearer GM. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. J Clin Immunol 1989; 84:1892-99

26. Redfield, RR, Wright, DC, Tramont, EC. The Walter Reed staging classification for HTLV-III/LAV infection. N Engl J Med 1986; 314:131-32

27. Hudecz F, Gonczol E, Plotkin SA. Preparation of highly purified human cytomegalovirus envelope antigen. Vaccine 1985; 3:300-304

28. Kantor GL, Goldberg LS, Johnson BL, Derechin MM, Barnett EV. Immunologic abnormalities induced by postperfusion cytomegalovirus infection. Ann Intern Med 1970; 73:553-58

29. Balachandran N, Oba DE, Hutt-Fletcher LM. Antigenic cross-reactions among herpes simplex virus types 1 and 2, Epstein-Barr virus, and cytomegalovirus. J Virol 1987; 61:1125-35

30. Lange M, Buimovici-Klein E, Kornfeld H, Quilop MT, Pahwa S, Reddy M, Wong P, Phillips L, Kiyasu J, Moriarty M, Wechter S, Cooper LZ, Grieco MH. Prospective observations of viral and immunologic abnormalities in homosexual males. Ann NY Acad Sci 1984; 437:350-63

31. Winkelstein W Jr, Samuel M, Padian NS, Wiley JA, Lang W, Anderson RE, Levy JA. The San Francisco men's health study: III. Reduction in human immunodeficiency virus transmission among homosexual/bisexual men, 1982-86. Am J Public Health 1987; 76:685-89

32. Lawrence J. CMV infections in AIDS patients. Infect in Surg 1986; Oct: 603-10

33. Holmberg SD, Stewart JA, Gerber AR, Byers RH, Lee FK, O'Malley PM, Nahmias AJ. Prior herpes simplex virus type 2 infection as a risk factor for HIV infection. JAMA 1988; 259:1048-50

34. Eyster ME, Gail MH, Ballard JO, et al. Natural history of immunodeficiency virus infection in hemophiliacs: Effects of T-cell subsets, platelet counts and age. Ann Intern Med 1987; 107:1

35. Webster A, Cook DG, Emery VC, Lee CA, Grundy JE, Kernoff PBA, Griffiths PD. Cytomegalovirus infection and progression towards AIDS in haemophiliacs with human immunodeficiency virus infection. Lancet 1989; 2: 63-66

36. Laskin OL, Stahl-Bayliss CM, Kalman CM, Rosecan LR. Use of ganciclovir to treat serious cytomegalovirus infections in patients with AIDS. J Infect Dis 1987; 155:323-26

37. DiNubile MJ. Screening HIV-infected recipients of blood transfusions for CMV infection. N Engl J Med 1990; 323:1282-83

## APPENDIX 1

List of abstracts, presentations and publications resulting in part or totally from research funded by DAMD 17-86-C-6285 and DAMD 17-90-C-0012:

1. Smith, Kendall O. An International Perspective on AIDS, 1988 (Abstract) Academic-Industry Joint Conference, San Antonio, Texas; March, 1988; presented in section on Immune Deficiency and AIDS.

2. Smith, Kendall O. and Ratner, J. Cytomegalovirus as a candidate Cofactor in AIDS (Abstract) ASM Annual Meeting, Miami Beach, Florida, 1988.

3. Smith, Kendall O. et al., Cytomegalovirus as a cofactor in AIDS (Abstract) ASM Annual Meeting, Anaheim, California, 1990.

4. Smith, Kendall O. et al., Cytomegalovirus is a cofactor associated with immunosuppression in HIV-infected subjects (Abstract) ASM Annual Meeting, Dallas, Texas, 1991.

5. Smith, Kendall O., Ludwig, M.J., Stigall, B.W. and Boswell, N. Enzyme-Linked immunosorbent assay (ELISA) for HIV antibody by a glass slide technique. J. Immunological Methods, J. Immunological Methods, 136 (1991) 239-246.

6. There are three other papers in revision for expected acceptance by journals, PI will notify DD as they become accepted.

## APPENDIX 2

Personnel receiving pay under contract support:

Janie Ludwig

Bernadette Wade

Christopher Hunter

Brian Stigall

Margaret Coughlin

Renee Brown

Donald Bruun

Frank Roach

Oredius Pressley

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